Science Arena Publications
Specialty Journal of Biological Sciences
Available online at www.sciarena.com
2017, Vol, 3 (1): 7-18

A study on probiotic properties of indigenous yeasts isolated from curd

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Abstract: Yeasts isolated from local dairy products were tested for their probiotic potential. The yeasts were exposed to different environmental conditions which are normally found to be in human gastrointestinal tract and were allowed to utilize different sugars so that they can be identified on the basis of biochemical characteristics. Yeasts were identified as belonging to genera *Kluyveromyces*, *Saccharomyces* and *Schwanniomyces* on basis of their ability to ferment sugars, assimilate carbon compounds and growth pattern in different conditions. Two of these yeasts were also identified using molecular methods. Among yeasts isolated, yeast belonging to *Klyuveromyces* sp. displayed excellent probiotic potential as compared to *Saccharomyces* sp. and *Schwanniomyces* sp. Probiotic properties that were studied and found appropriate for yeasts from *Kluyveromyces* sp. include bile tolerance, acid tolerance, cell surface hydrophobicity and certain other characteristics. All the characteristics of yeasts related to probiotic potential were also compared to yeast *Saccharomyces boulardii* which is utilized as probiotic supplement nowadays.

Keywords: Probiotic, Yeast, Dairy products, *Kluyveromyces marxianus*, *Saccharomyces boulardii*

INTRODUCTION

As per the joint FAO/WHO expert consultation, live microorganisms which confer health benefit to the host on administrating in adequate amount can be considered as 'Probiotics' (FAO/WHO, 2002). Extensive work had been carried out for finding out probiotic properties of various bacterial species like *Lactobacillus* sp., *Bifidobacterium* sp., *Lactococcus* sp., *Enterococcus* sp., *Streptococcus* sp., *Pediococcus* sp., *Leuconostoc* sp. etc. and reviewed (Fijan, 2014). Nowadays, awareness regarding the close relationship between health and diet is increasing due to which special attention is given to functional properties of probiotics. There are lots of dairy products and fermented products which are found to be good source for isolation of probiotics. Lot attention has been paid to probiotic properties of bacteria but yeasts were given lower preference till now as compared to bacteria. Some of the yeasts and their products are generally regarded as safe for human consumptions (Reed and Nagodawithana, 1991). Yeasts can be easily isolated from dairy and bakery products. In recent

days, a yeast *Saccharomyces boulardii* is becoming available as probiotic supplement in pharmaceutical preparations after extensive studies related to its beneficiary effects (Czerucka *et al*., 2007).

In present study, probiotic characterization of indigenous yeasts isolated from curd was carried out and their probiotic potential was evaluated. As suggested by FAO/WHO, sugar fermentation profiling (a pattern generated on fermentation of sugars) is one of the key phenotypes to be investigated for identification purpose. Hence, the sugar fermentation profile was also studied. The probiotic properties of these indigenous yeasts were also compared with the probiotic yeast strain *Saccharomyces boulardii*. Objective of the study was to search for probiotic potential of the yeasts found in local dairy products such as curd.

MATERIALS AND METHODS

Isolation and maintenance of yeasts

The yeasts were isolated from curd samples obtained from various locations of Gujarat, India as explained earlier (Shah & Parikh, 2016). The yeast was grown initially on YPD medium composed of w/v each of 1 g% yeast extract, 2 g% peptone and 2 g% D-glucose. For the purpose of maintenance, YPD agar containing 2% w/v starch (YPDS) was used (Sherman, 2002). Agar powder (3 g% w/v) was used as solidifying agent wherever required. Respective yeast was grown in YPD broth till the optical density of the medium reaches to 1.0 at 600nm against sterile YPD broth as blank and was used freshly as an inoculum. For purpose of study, four isolated yeast strains and two previously identified strains were used.

Identification of yeasts

Ability of individual yeasts to ferment various sugars, assimilate various carbon sources and grow in various conditions were used for their identification as explained by Kurtzman *et al*. (2011). In addition, two of the yeasts were identified on the basis of sequence of 18s rDNA (ITS).

Sugar fermentation profile

Yeasts can be characterized on the basis of biochemical reaction, in order to ascertain their identification at various lengths. Yeasts were characterized according to their fermentation profiles for ability to ferment different sugars. Each sugar solutions were prepared at a final concentration of w/v each of 2.0 g% respective sugar, 0.45 g% yeast extract, and 0.75 g% peptone and it contained bromothymol blue as the indicator. Sugars were prepared as concentrated solutions and filter sterilized separately with 0.22µ filter membrane. Medium

without sugar was sterilized in autoclave followed by addition of filter sterilized concentrated sugar solution (Wickerham, 1951). The turbidity and the color change from Blue to yellow were recorded as positive fermentation results for acid and compared with the positive and negative controls. Culture that utilized sugar, produced acid which changed the color of broth from Blue to yellow.

Preparation of yeast for inoculum and measurement of degree of growth

Yeasts were grown overnight in YPD broth and used to inoculate respective medium. Assessment of degree of growth was carried out by directly observation of tubes by eyes. The tubes were shaken to disperse the yeast cells and the growth was observed.

Growth at various pH

YPD broth with acidic pH 3.0 and alkaline pH 9.0 were inoculated with overnight grown yeast at rate of 1% followed by incubation at 30 ˚C for 24-72 hours as explained by Satyajit *et al.* (2016). The growth was assessed by observing the turbidity of the culture broths as explained above.

Growth at different NaCl concentrations

Growth of yeasts in the presence of 4% and 6.5% NaCl was studied by using YPD broths containing respective NaCl concentrations (Satyajit *et al.*, 2016). These tubes were inoculated with 1% fresh inoculum and then incubated at 30 ˚C for 24-72 hours and visually examined on the basis of turbidity of the culture.

Growth in 0.1% Methylene blue milk

Methylene blue milk (prepared in skimmed milk) was prepared in screw cap tube and autoclaved. Later it was inoculated with the test culture of isolate followed by incubation at 30 ˚C. During incubation visual examination for reduction in the color of broth was carried out.

Growth in presence of bile

YPD broth containing w/v 10 and 4% bile was inoculated with 1% fresh inoculum and incubated for 24-72 hours. Growth of the isolates on bile salt in respective broths was recorded on the basis of turbidity.

Growth in 0.04% Azide

YPD broth containing 0.04% azide were inoculated with 1% fresh inoculum and incubated at 30 ˚C for 24-72 hours and visually examined on the basis of turbidity of the culture.

Acid and bile tolerance

Yeasts were grown in YPD broth till its optical density reaches 1.0 at 600nm against sterile YPD broth as blank. Yeast biomass was collected by centrifugation of yeast grown in YPD broth. Pellets obtained by centrifugation of the culture broth at 8000 rpm for 15 min were suspended in N-saline to the original volume. Acid tolerance was assessed at two different pH values where pH of N-saline was adjusted with 0.5% HCl (Zheng *et al.*, 2013). Bile tolerance was tested at 0.5 and 1.0% concentrations where cells were resuspended in N-saline containing respective concentration of bile (Zheng *et al.*, 2013). Sampling was carried out on hourly incubation and the cells were counted in Neubauer's chamber after carrying out necessary dilution. Living yeasts were counted on the basis of vital staining (Mochaba *et al.*, 1998).

$$\% \text{ survivability} = (\text{No. of cells at } N^{th} \text{ hour} / \text{No. of cells at } 0^{th} \text{ hour}) \times 100$$

Cell surface hydrophobicity

The in vitro cell surface hydrophobicity was determined by observing yeast adherence to hydrocarbon assay modified from the methods of Rosenberg *et al.* (1980). Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the initial aqueous yeast suspension due to cells partitioning into a hydrocarbon layer. Hydrophobicity was calculated from three replicates as the percentage decrease in the optical density of the initial aqueous yeast suspension due to cells partitioning into a hydrocarbon layer.

ß-galactosidase production

The ß-galactosidase activity of yeast cultures were determined by the method that quantifies the enzyme activity without disrupting the cell and utilizes o-nitrophenyl-ß-D galactopyanoside (ONPG) as substrate (Lederberg, 1950; Miller, 1972). The development of Yellow color indicated positive test for ß-galactosidase.

RESULTS AND DISCUSSION

Identification of yeasts

Ability of yeasts to ferment various sugars and assimilate various carbon sources was assessed and the results are displayed in Table 1. On the basis of biochemical characterization, HYE26 and HYE30 were found to be belonging to genus *Saccharomyces* sp, and *Schwanniomyces* sp. respectively. Both yeasts, HYE14 and HYE54 were found to be from genus *Kluyveromyces* sp.

Table 1a. Sugar fermentation profile of yeast isolates

	HYE14	HYE26	HYE30	HYE54
Glucose	+	+	+	+
Galactose	+	-	+	+
Sucrose	+	+	+	+
Maltose	+	+	+	+
Lactose	+	-	+	+
Raffinose	+	-	-	+
Trehalose	-	-	-	-

Table 1b. Assimilation of carbon sources by yeasts

	HYE14	HYE26	HYE30	HYE54
Glucose	+	+	+	+
Inulin	+	-	+	+
Sucrose	+	+	+	+
Raffinose	+	-	+	+
Melibiose	-	-	-	-
Galactose	+	-	+	+
Lactose	+	-	+	+
Trehalose	-	+	+	-
Maltose	+	+	+	+
Melezitose	-	+	+	-
Cellobiose	-	-	+	-
D-Xylose	+	-	+	+
D-Ribose	-	-	-	-
Glycerol	+	+	+	+
D-Mannitol	-	+	+	-

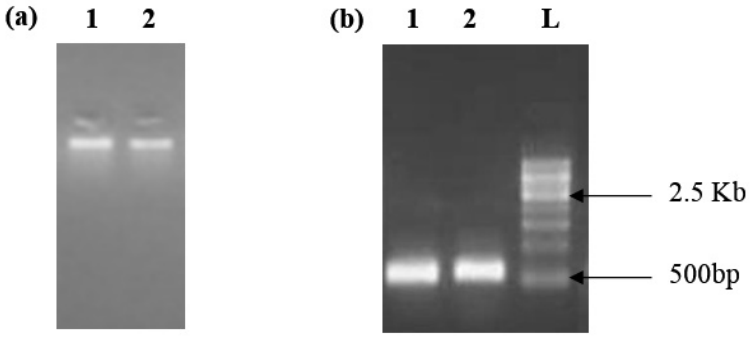
Table 1c. Growth of yeasts in different conditions

	HYE14	HYE26	HYE30	HYE54
50% Glucose	-	+	-	-
Gelatin liquefaction	-	-	-	-
Growth at 19 ˚C	+	+	+	+
Growth at 25 ˚C	+	+	+	+
Growth at 30 ˚C	+	+	+	+
Growth at 35 ˚C	+	+	+	+
Growth at 37 ˚C	+	+	+	+
Growth at 40 ˚C	+	+	+	+
Growth at 45 ˚C	+	+	-	+

Identification of Yeasts HYE14 and HYE54 was also carried out on the basis of sequence of 18s ribosomal DNA. On PCR amplification of 18S rDNA from yeast (HYE14 and HYE54) genomic DNA, ~ 680 bp long fragment was obtained (Fig. 1) which was followed by its sequencing. The sequence was found to be having 99% homology with *Kluyveromyces marxianus* sp. showing maximum score 1197 with 0.0 *e*-value for both. Yeasts HYE14 and HYE54 are described as *Kluyveromyces marxianus* HS01 and *Kluyveromyces marxianus* HS02 respectively. The sequence was submitted to GenBank, NCBI (Accession no. KY073321 and KY073322 respectively for HYE14 and HYE54).

Figure 1a. DNA isolated from Lane 1: HYE14 and Lane 2: HYE54

Figure 1b. Lane 1: PCR product, HYE14; Lane 2: PCR product, HYE54 and Lane 3: Marker



Growth in presence of NaCl, methylene blue milk, bile and azide

Both yeasts belonging to *Klyuveromyces* sp. grow in 4% NaCl only while growth in both NaCl concentrations was observed for all remaining yeasts. All the yeasts were able to grow in 0.1% methylene blue milk which resulted in decolourization of milk due to oxidative activities of yeast followed by curdling of milk. 1% and 4% bile were not able to restrict growth of yeast isolates when provided in nutrient medium. Sodium azide is a useful preservative in hospitals and laboratories which is important in bulk reagents and stock solutions which may otherwise support bacterial growth where the sodium azide acts as a bacteriostatic by inhibiting cytochrome oxidase in Gram-negative bacteria (Lichstein *et al.*, 1943). The yeasts were found to be able to grow in presence of azide. The detailed results are shown in Table 2.

Table 2. Probiotic properties of yeasts

No	Characteristic	*Klyuveromyces marxianus* HS01	*Klyuveromyces marxianus* HS02	*Schwanniomyces* sp.	*Saccharomyces* sp.	*Saccharomyes boulardii*	*Saccharomycopsis fibuligera*
1	pH 2.0	+	+	+	+	+	+
2	pH 3.0	+	+	+	+	+	+
3	pH 9.0	+	+	+	+	+	+
4	6.5% NaCl	-	-	+	+	+	+
5	4% NaCl	+	+	+	+	+	+
6	1% Bile	+	+	+	+	+	+
7	4% Bile	+	+	+	+	+	+
8	0.04% Azide	+	+	+	+	+	+
9	0.1% Methylene blue	+	+	+	+	+	+
10	ß-Galactosidase	+	+	+	-	+	-

Tolerance against acid and bile

Bile is a part of gastrointestinal system where it is present in 0.5 to 2.5% concentrations. Hence, bile tolerance is considered as main prerequisite for growth and survival in gastrointestinal tract (Liong & Shah, 2005). *Saccharomyces boulardii* is a well-known for its probiotic properties (Łukaszewicz, 2012) displayed excellent bile resistance to both concentrations of bile tested. *Kluyveromyces marxianus* HS01 and *Kluyveromyces marxianus* HS02 displayed remarkable tolerance to 0.5% bile in initial hours which ended with zero at 6 hours. *Saccharomyces* sp. showed low bile tolerance and *Schwanniomyces* sp. was not able to tolerate bile more than two hours when bile concentration was 0.5%. At 1% bile, all yeasts tolerated it up to 4 hours except *Saccharomyces* sp. and *Schwanniomyces* sp. Acid tolerance is one of the important properties of probiotics. Probiotic microorganisms carries ability to resist acid via many mechanisms which includes higher cytoplasmic buffering capacity of activity of membrane ATPases (Rius *et al.*, 1994). All the yeasts shown remarkable acid tolerance at pH 2.0 which was found to be reduced when tested at pH 1.5. Here, acidic pH can be considered homologous to stomach (Shukla *et al.*, 2010) which directly indicates the sustainability of yeasts when ingested and gets exposure to digestive environment in stomach.

Figure 1. Bile tolerance by yeasts (0.5% bile)

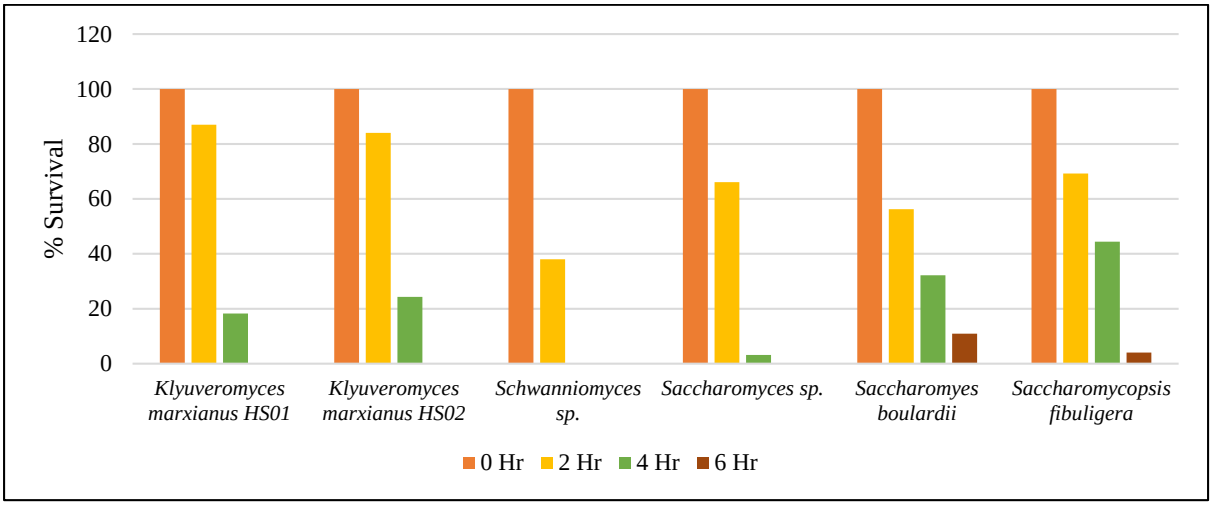


Figure 2. Bile tolerance by yeasts (1% bile)

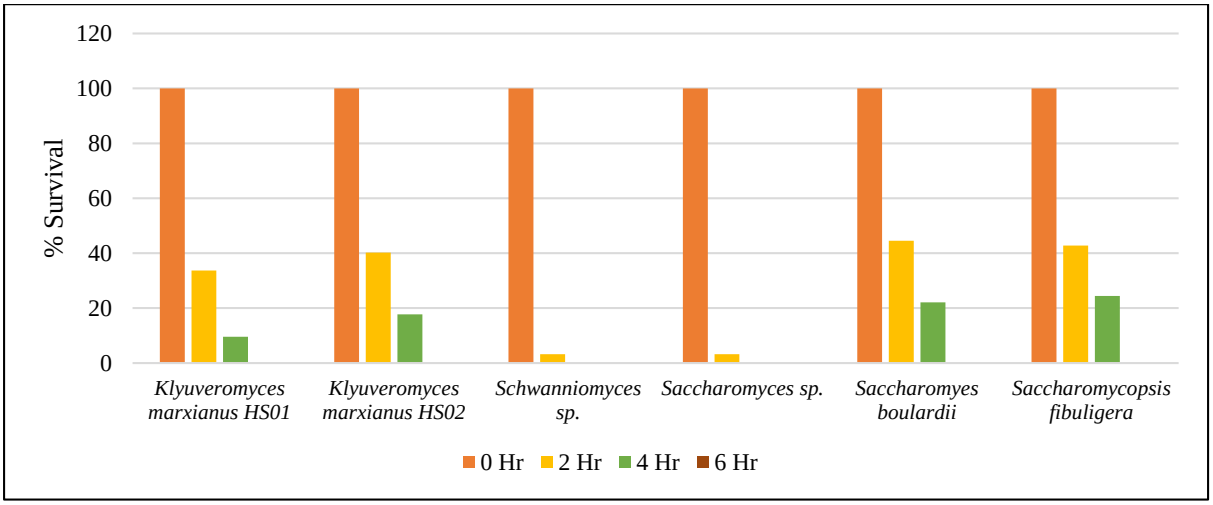


Figure 3. Acid tolerance by yeasts (pH 2.0)

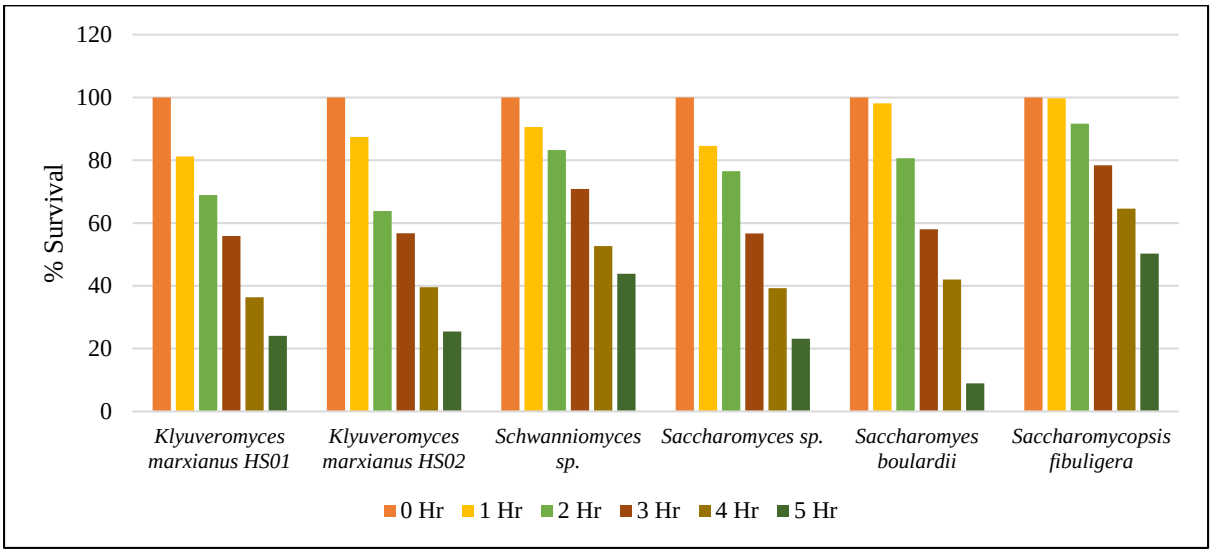
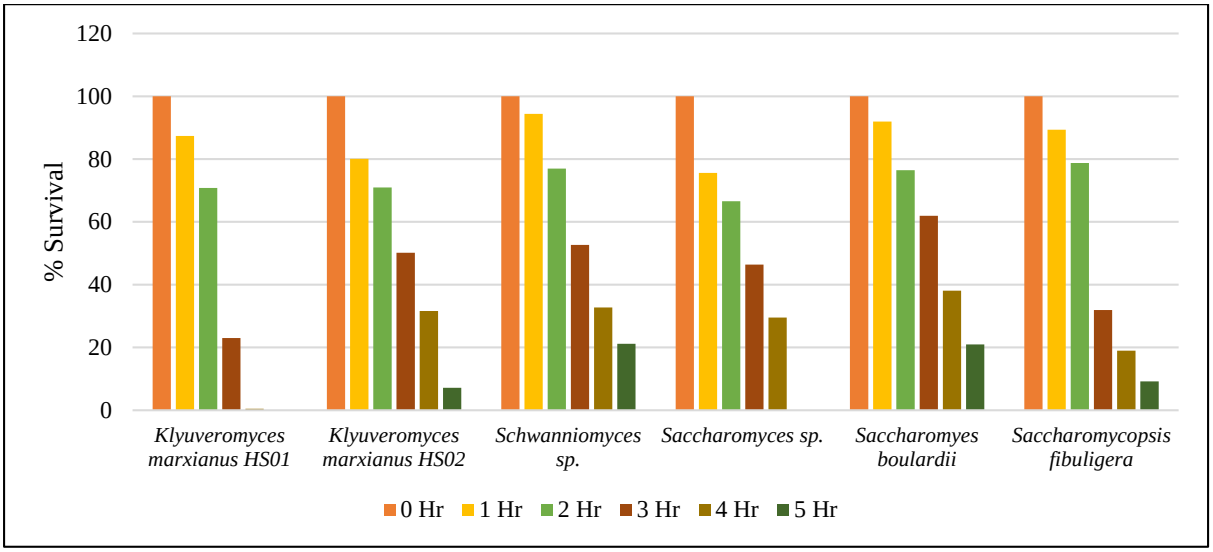


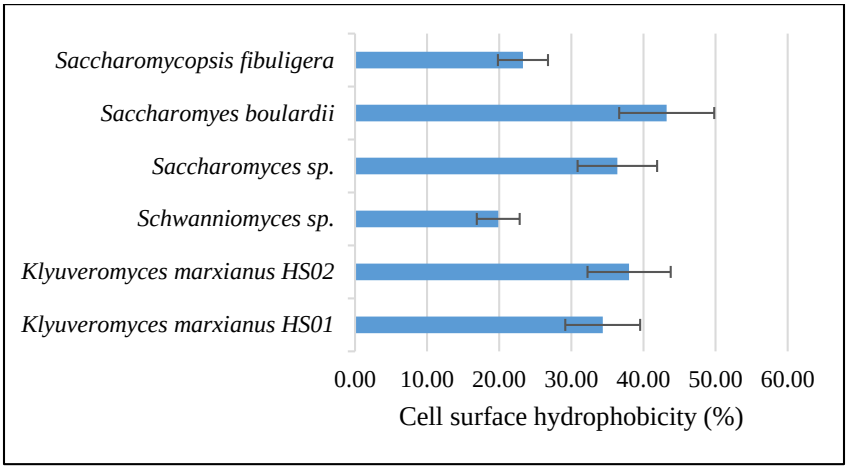
Figure 4. Acid tolerance by yeasts (pH 1.5)



Cell surface hydrophobicity and production of ß-galactosidase

Saccharomyces boulardii shown maximum cell surface hydrophobicity which indicates its ability to adhere intestinal tract. One of the mechanism of probiotic is prevention of bacterial adherence which can be accomplished by adherence of yeasts to intestinal mucosa and prevention of potentially harmful bacteria to adhere (Moslehi-Jenabian *et al*., 2010). In our experiments, *Saccharomyces* sp. and both yeasts belonging to *Kluyveromyces* sp. exhibited remarkable cell surface hydrophobicity.

Figure 5. Cell surface hydrophobicity of yeasts



CONCLUSION

Beneficial aspects of probiotic strains can be expected only when they are able to survive passage through the human stomach, digestive system and colonize the human gut. All the isolates displayed good tolerance to conditions which are generated on exposure to acid as in stomach. In addition, they also exhibited remarkable tolerance to bile as in intestine. But, on having eye at other properties among all four isolates on comparison with *Saccharomyces boulardii*, yeasts belonging to *Klyuveromyces* sp. exhibited competitive potential as probiotic candidates since they displayed good tolerance to bile and acid which are normally found in gastrointestinal tract. In addition, they also carried good cell surface hydrophobicity which can provide healthy competition to potentially harmful bacteria to adhere intestine. Production of ß-galactosidase is also a unique feature of *Klyuveromyces* sp. which can be important remedy in relieving disorders like lactose intolerance.

REFERENCES

Czerucka D, Piche T, Rampal P. 2007. Review article: yeast as probiotics–*Saccharomyces boulardii*. Alimentary pharmacology & therapeutics. 26(6):767-778.

Fijan S. 2014. Microorganisms with claimed probiotic properties: an overview of recent literature. International journal of environmental research and public health. 11(5):4745-4767.

Joint FAO/WHO working group. 2002. Report on Drafting Guidelines for the Evaluation of Probiotics in Food. In: Guidelines for the Evaluation of Probiotics in Food London Ontario Canada: Joint FAO/WHO working group meeting.

Kurtzman CP, Fell JW, Boekhout T, Robert V. 2011. Methods for isolation phenotypic characterization and maintenance of yeasts. In: The yeasts a taxonomic study 5th edn. Elsevier, Amsterdam.

Lederberg J, 1950. The beta-d-galactosidase of *Escherichia coli* strain K-12. Journal of bacteriology. 60(4):381.

Lichstein J, Solis-Cohen LEON. 1943 Familial tuberous sclerosis epiloia without adenoma sebaceum: report of two cases. Journal of the American Medical Association. 122(7):429-432.

Liong MT, Shah NP. 2005. Acid and bile tolerance and cholesterol removal ability of lactobacilli strains. Journal of dairy science. 88(1):55-66.

Łukaszewicz M. 2012. *Saccharomyces cerevisiae* var *boulardii*-Probiotic Yeast. INTECH Open Access Publisher.

Miller J. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, New York.

Mochaba F, O'Connor-Cox ESC, Axcell BC. 1998. Practical procedures to measure yeast viability and vitality prior to pitching. Journal of the American Society of Brewing Chemists. 56(1):1-6.

Moslehi-Jenabian S, Lindegaard L, Jespersen L. 2010. Beneficial effects of probiotic and food borne yeasts on human health. Nutrients 2(4):449-473.

Reed G, Nagodawithana TW. 1991. Yeast-derived products. In: Yeast technology. Springer Netherlands.

Rius N, Solé M, Francia A, Lorén JG. 1994. Buffering capacity and membrane H^+ conductance of lactic acid bacteria. FEMS microbiology letters. 120(3):291-295.

Rosenberg M, Gutnick D, Rosenberg E. 1980. Adherence of bacteria to hydrocarbons: a simple method for measuring cell-surface hydrophobicity. FEMS microbiology letters. 9(1):29-33.

Satyajit S, Balaram M, Dutta HS, Tripathy TT. 2016. Isolation and characterization of probiotic Lactobacillus species from curd samples and evaluation of their antagonistic potential. Journal of Microbiology, Biotechnology and Food Sciences. 6(3):867-873.

Shah H, Parikh S. 2016. Selection of yeasts from local dairy products for the production of glucoamylase. IJIRMF. 2(7):203-208.

Sherman F. 2002. Getting started with yeast. In: Methods in Enzymology. Academic Press.

Shukla G, Sharma G, Goyal N. 2010. Probiotic characterization of lactobacilli and yeast strains isolated from whey beverage and therapeutic potential of Lactobacillus yoghurt in murine giardiasis. Am J Biomed Sci. 2:248-261.

Wickerham LJ. 1951. Taxonomy of yeasts (No 1029). US Dept. of Agriculture.

Zheng Y, Lu Y, Wang J, Yang L, Pan C, Huang Y. 2013. Probiotic properties of Lactobacillus strains isolated from Tibetan kefir grains. PloS one. 8(7):e69868.